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STAIN TECHNOLOGY

OCTOBER, 1931

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SUGGESTIONS TO AUTHORS SUBMITTING MANUSCRIPT

Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

To be accepted for publication, manuscript should be original typewritten copy (not carbon copy) either double or triple spaced, with wide margins. Special attention should be given to the headings of tables and to legends for illustrations. These in all cases should be complete in themselves, so as to make the table intelligible to anyone studying it, without consulting the text of the article. In the case of complicated tables, typewritten manuscript is not absolutely required, provided all words and figures are carefully written or printed in a legible hand. All references to literature must be carefully verified and in ordinary cases the complete title and reference should be given.

Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

All illustrations should accompany the manuscript and should be numbered with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. Line drawings must be made in India ink. Photographs for half-tones must be on paper giving black and white tones and should preferably have a glossy finish. *Colored illustrations accepted only at author's expense.*

Authors are especially urged to make all staining formulae definite and accurate. If known to the author, the dye content of the stain should be given and the exact dye used should be specified. If this information is not known, the name of the manufacturer should be stated and that of the stain itself exactly as worded on the label. Certified stains should be specified by certification number.

STAIN TECHNOLOGY

VOLUME VI OCTOBER, 1931 NUMBER 4

PROGRESS IN THE STANDARDIZATION OF STAINS

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stain approved since the last one listed in the April number of this journal.

STAINS CERTIFIED MARCH 1 TO AUGUST 31, 1931*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission	Date approved
Methyl orange	NM 4	94%	As histological counter-stain	Mar. 27, 1931
Methylene blue tablets	NA 6	0.9%	For reductase test in milk	Apr. 2, 1931
Crystal violet	NC 11	94%	As histological and bacteriological stain	Apr. 4, 1931
Brilliant green	NBg 4	93%	For use in bacteriological media	Apr. 18, 1931
Erie Garnet B	NGe 1	68%	As histological stain	Apr. 18, 1931
Methylene blue	CA 12	80%	As histological and bacteriological stain	May 1, 1931
Methylene blue	CA 13	83%	As bacteriological stain	May 1, 1931
Basic fuchsin	CF 12	91%	For staining and for use in Endo Medium	May 1, 1931
Bismarck brown	CN 3	50%	As histological stain	May 1, 1931
Orange G.	LO 3	77%	As histological stain	May 1, 1931
Crystal violet	NC 12	94%	As histological and bacteriological stain	May 8, 1931
Thionin-eosinate mixture	ATe 1	....	In Geschickter stain for frozen tissue	May 18, 1931
Azure-Erie-garnet	AAg 1	....	In Geschickter stain for frozen tissue	May 18, 1931
Acid fuchsin	NR 9	57%	As histological stain and in Andrade indication	May 23, 1931
Erythrosin B	NEr 2	88%	As histological stain	May 25, 1931
Methyl green	NG 8	57%	As histological stain and in Pappenheim technic	June 1, 1931
Congo red	NQ 4	90%	As histological stain	June 16, 1931
Hematoxylin	FH 9	....	As histological stain	June 24, 1931
Safranin O	LS 4	68%	As histological and cytological stain	July 6, 1931
Safranin O	NS 8	92%	As histological and cytological stain	July 6, 1931
Brilliant green	CBg 4	90%	For use in bacteriological media	July 10, 1931
Erie garnet B	NGe 2	76%	As histological stain	July 20, 1931
Basic fuchsin	LF 1	73%	For staining and in Endo medium	Aug. 27, 1931
Azure A	NAz 4	80%	As histological stain and as constituent of blood stains	Aug. 27, 1931
Toluidine blue	NU 2	....	As histological stain	Aug. 27, 1931
Bismarck brown	LN 1	58%	As histological stain	Aug. 27, 1931

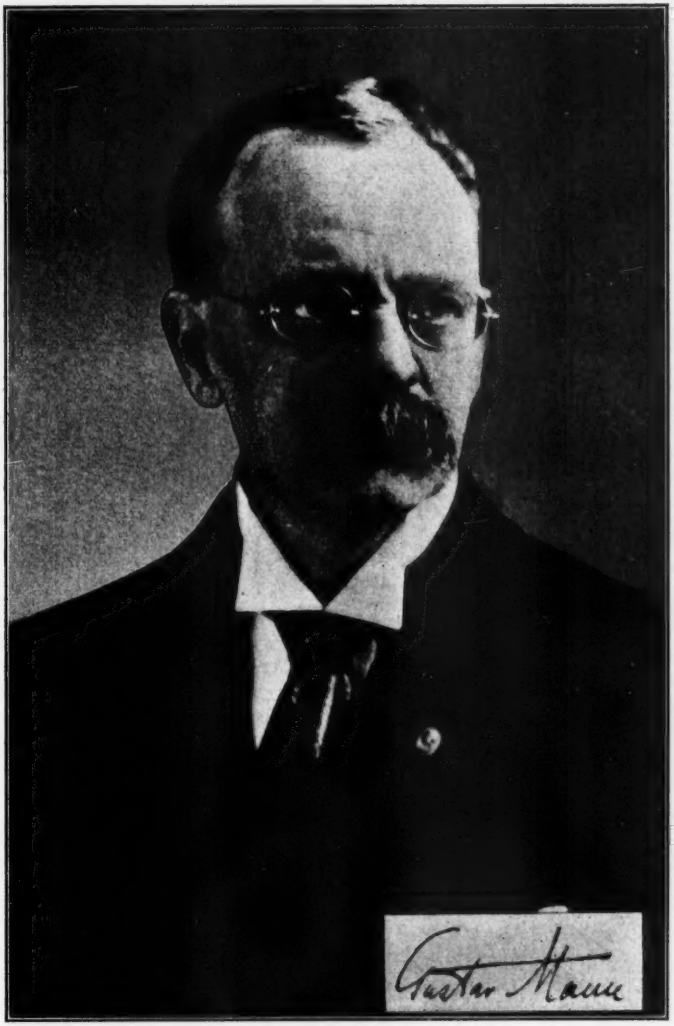
*The name of the company submitting any one of these dyes will be furnished on request.

PORTRAIT OF GUSTAV MANN

In an early number of this journal a brief account[1] was published of the life and work of the histologist, Gustav Mann. At the time no photograph of Dr. Mann was available, or one would have been included with the article. As a splendid portrait of him has now been furnished us, we are publishing it for the interest of our readers.

Dr. Mann, it will be recalled, was a prominent histologist at Oxford during the early years of this century. His book, Physiological Histology (Oxford, 1902) was the first biological text book to include a comprehensive discussion of the chemistry of dyes. His book was of immense value to the Stain Commission at the beginning of its activities in this country.

[1]By Lloyd Arnold. Stain Tech. 2, 4–7. 1927.



DR. GUSTAV MANN
1864–1921

THE SMEAR TECHNIC IN PLANT CYTOLOGY

KARL SAX

Arnold Arboretum, Harvard University, Jamaica Plain, Massachusetts

ABSTRACT.—**The following method of making permanent smears of pollen mother cells is in general use and gives excellent results. Determine the stage of meiosis from aceto-carmin mounts. Smear the pollen mother cells on a dry slide. Fix in Navaschin's or a modified Flemming's solution from 1 to 2 hours. Wash in 10 to 20% alcohol from 15 to 30 minutes. Stain in 1% aqueous crystal violet from 1 to 5 minutes. Rinse in water and pass thru 30 to 50% alcohol, about 15 to 20 seconds in each. Transfer to 80% alcohol containing 1% iodine and 1% potassium iodide for 30 seconds. Destain with absolute alcohol, followed by clove oil, xylol, balsam and cover.**

Permanent smears for chromosome counts can be quickly made by smearing pollen mother cells on a dry slide, fix and stain with aceto-carmin, dehydrate with mixtures of absolute alcohol and acetic acid, follow with xylol, balsam, and cover.

The smear technic in plant cytology has made possible a more critical study of chromosome structure and has facilitated chromosome counts in numerous genera. Permanent smear preparations are far superior to the usual sectioned material for a study of the internal morphology of the chromosomes and their method of association at meiosis. Pollen mother cells are rapidly fixed since they come into direct contact with the fixative and the material is not subjected to a long period of dehydration and the high temperature necessary with the paraffin method. The aceto-carmin technic offers an easy and rapid method for obtaining chromosome counts and may be useful for critical studies of the earlier stages of meiosis.

ACETO-CARMIN SMEARS

The aceto-carmin smear technic as developed by Belling[1] (1926) has been successfully used for obtaining chromosome counts by numerous investigators in this country and abroad. Properly prepared aceto-carmin mounts are often superior to sectioned material for a study of the chromosome number at meiosis because there is no clumping of the chromosomes and the pollen mother cells are intact.

[1]Belling, J. The iron-aceto-carmin method of fixing and staining chromosomes. Biol. Bull., 50, 160–162. 1926.

The iron-aceto-carmin solution is prepared by mixing equal proportions of glacial acetic acid and water. An excess of powdered carmin is added and the solution is heated until it begins to boil. It is then cooled and filtered. To this solution is added a few drops of a mixture of ferric hydrate in 50% acetic acid or the iron can be added by stirring the solution with a file or an iron rod. Enough iron should be added to act as a mordant, but too much produces an undesirable precipitate. The solution appears to have a tinge of purple when the proper amount of iron is present. For some material this solution can be diluted with 50% acetic acid with good results.

The anthers, if small, are placed on a slide in a drop of aceto-carmin and crushed with the end of another slide or with a flat needle. The anther debris is removed and the preparation is covered. When the anthers are large, the ends are cut off and the pollen mother cells are pressed out into the drop of aceto-carmin or the pollen mother cells may be smeared on a dry cover glass which is quickly dropped on the aceto-carmin on the slide. Chromosome counts can frequently be made at once, but the best preparations are obtained after the pollen mother cells have been in the aceto-carmin for one to several days. To prevent the evaporation of the aceto-carmin, the cover glass should be sealed with balsam or with a mixture of equal parts of paraffin and gum mastic which has been heated, thoroly mixed, and cooled. This mixture is best applied with a hot wire which is placed in the paraffin gum mastic and placed along the edge of the cover. After a day or two a slight pressure on the cover glass will break open the pollen mother cell so that the chromosomes will be flattened out in the same plane.

Better results are often obtained if the anthers are first fixed in a mixture of 3 parts absolute alcohol and 1 part of absolute acetic acid from 1 to 24 hours. The alcohol seems to act as a mordant so that the chromosomes take a dark stain. If the anthers are to be kept for later examination they should be transferred to 70% alcohol after fixing in the acetic acid and alcohol for 12 to 24 hours. Aceto-carmin preparations can be made from the anthers preserved in 70% alcohol at any time.

Preparations made with a solution containing a minimum of iron will often keep for months if well sealed. More permanent preparations can also be made by mounting the pollen mother cells in balsam. The method for making permanent mounts of aceto-carmin smears described by McClintock[2] (1929) is the best one for general use. The

[2]McClintock, B. A method for making aceto-carmin smears permanent. Stain Techn., 4, 53–56. 1929.

slide is heated over an alcohol flame for about a second and repeated several times so that the cells are somewhat flattened. An electric hot plate may also be used. The aceto-carmin solution should not be boiled. The slide is then placed in a petri dish containing a 10% solution of acetic acid and the cover glass floated off. Some of the pollen mother cells will stick to the cover and some to the slide so that both should be transferred to a staining jar containing equal parts of absolute alcohol and acetic acid. After several minutes, transfer thru the following solutions for several minutes each: 1 part acetic acid and 3 parts absolute alcohol, 1 part acetic acid and 9 parts absolute alcohol, absolute alcohol, equal parts absolute alcohol and xylol, and finally xylol. Recombine the slide and cover glass in xylol-balsam.

Permanent aceto-carmin mounts can also be made by smearing the pollen mother cells on a dry slide, fix and stain with aceto-carmin, and dehydrate with mixtures of absolute alcohol and acetic acid as outlined in the preceding paragraph. If necessary the preparation can be mounted in aceto-carmin for several days before dehydrating and mounting in balsam. In a recent issue of STAIN TECHNOLOGY Steere[3] (1931) suggests that smears be fixed in hot aceto-carmin from 1 to 10 minutes and then dehydrated.

Somatic chromosomes can also be fixed and stained with the aceto-carmin technic. Heitz[4] (1926) has been able to get numerous figures with a modification of this method. Root tips are fixed for several minutes in a hot solution of 3 parts absolute alcohol and one part glacial acetic acid. They are then transferred to a drop of aceto-carmin on a slide, cut, and teased apart. The preparation is covered and left for several hours or even a day until the tissue is deeply stained. The slide is then heated until the cytoplasm is destained. A slight pressure on the cover glass will spread the cells apart so that frequently the tissue is only one cell thick and often isolated cells can be found containing division figures. Such preparations show the longitudinal split at metaphase, the spindle fiber constrictions and trabants.

The writer has found it difficult to get the somatic cells properly oriented for a study of the chromosome number and often many root tips are required before satisfactory figures are obtained with this method.

The aceto-carmin technic is not only good for a study of chromo-

[3]Steere, W. C. A new and rapid method for making permanent aceto-carmin smears. Stain Techn. 6, 107–111. 1931.

[4]Heitz, E. 1926. Der Nachweis der Chromosomen. Zts. f. Bot., 18, 625–681. 1926.

some numbers, but it is also often suitable for the investigation of chromosome structure. Belling has obtained prophase stages of meiosis which show the chromomeres and Miss McClintock's preparations of pachytene chromosomes in Zea are excellent. It is also possible to show the coiling of the chromonemata at the first metaphase of the reduction division in large chromosomes. However, for the finer details of the structure of metaphase chromosomes the permanent smears give better results.

Permanent Smears

The condition of the pollen mother cells is quickly obtained from temporary aceto-carmin preparations. After some experience the proper stages for divisions can be determined from the appearance of the anthers. Small anthers are placed on a clear slide and quickly smeared with a scalpel, a section lifter, or another slide. The slide is then immediately inverted in the fixing fluid. Large anthers should be cut into segments. According to Belling[5] (1930) it is essential to dry the anther segments with blotting paper so that the pollen mother cells do not come in contact with the cell sap. When a considerable mass of pollen mother cells can be squeezed from the anther it is often necessary to smear these cells lightly. The writer has obtained good results with *Secale* by smearing one anther at a time. About a dozen anthers are placed on a slide. The end of an anther is cut off and the contents squeezed out with a flat needle. The anther is then pulled away from the pollen mother cells so that they are pulled out into long strings on the slide. A drop of the fixing solution is quickly but carefully dropped on the pollen mother cells. When all of the anthers are smeared the slide is placed in the tray of fixing solution.

The fixative is placed in a surgeon's tray about 8 inches long, 3 inches wide and 1½ inches deep (Will Corporation). On the bottom of the tray there are three corrugations which support the slides. Forty cubic centimeters of fixing solution are sufficient for four slides.

The best general fixative seems to be Navaschin's solution which is essentially the same as Allen's B 15 without the picric acid. This solution is made up in two parts. Solution A contains 1.5% chromic acid and 12% acetic acid. Solution B is made up of equal parts of water and commercial formalin. According to Belling, solution B should contain about one part formalin to 3 of water for metaphase chromosomes. Solutions A and B are mixed in equal proportions just before using.

[5]Belling, J. The use of the microscope. McGraw-Hill Co., New York. 1930.

Darlington has obtained good results with a modified Flemming's solution made up as follows: 60 cc. of 1% chromic acid, 20 cc. of 2% osmic acid, and 25 cc. of 10% acetic acid.

According to Taylor[6] (1924), who first described this technic for plant material, the pollen mother cells should be fixed for about 15 minutes, but most cytologists leave the slides in the fixing solution for several hours. The shorter time gives sufficient fixation for chromosome counts and general morphology of the chromosomes but a longer fixation, from one to two hours, seems to be better for a study of chromosome structure.

After fixing, the slides are washed in tap water from 15 to 30 minutes. Only one change of water is necessary. At this time fragments of anther walls and any other somatic tissue are removed with forceps. In many cases better results are obtained if the slides are quickly rinsed in water and transferred directly to 20% alcohol for 20 to 30 minutes.

If the material has been fixed with a solution containing osmic acid it is necessary to bleach the slides in a weak solution of hydrogen peroxide for about 20 minutes. If the preparations are placed in sunlight they will bleach faster.

The pollen mother cells may now be stained. Any standard staining technic gives good results when properly used. Dyes from various American sources have been employed. Newton's modification of the Gram stain is the most convenient and gives good results. The preparations are stained in a 1% aqueous solution of gentian violet or crystal violet which has been boiled and filtered. Stain from 1 to 5 minutes. Rinse in water and pass thru 30 and 50% alcohol, about half a minute in each. Transfer to 80% alcohol, containing 1% iodine and 1% potassium iodide, for 30 seconds. This solution sets and darkens the stain. Wash with absolute alcohol until the cytoplasm is free from stain and the chromosomes are properly differentiated. This usually requires about 30 seconds. Cover the slide with clove oil and examine under the microscope. If overstained the clove oil will often destain the material slowly, but usually it is necessary to wash again with absolute alcohol. The clove oil is replaced with xylol which is allowed to remain for about half a minute. The balsam and cover glass are then added.

Belling's (1930) brazilin stain has also given excellent results. After fixing, the slides are washed in 1 part of Navaschin's solution A in 3 parts water for about 10 minutes. The slides are then carried thru a series of alcohols, 15, 30 and 50% up to 70% alcohol, where

[6]Taylor, W. R. The smear for plant cytology. Bot. Gaz., 78, 236–238. 1924.

they remain overnight. They are then placed in a 1% solution of iron alum in 70% alcohol for 24 hours. Rinse and wash in 70% alcohol from 15 minutes to several hours. Stain in 0.5% solution of brazilin in 70% alcohol from 2 to 24 hours. Wash in 70% alcohol and destain in the alcoholic iron alum. When properly differentiated the slides are washed in alcohol, transferred to 95% alcohol and passed thru absolute alcohol, absolute alcohol and cedar oil in equal parts, xylol and cedar oil in equal parts, and finally xylol. The preparation is then mounted in immersion cedar oil. This technic could probably be shortened considerably.

The hematoxylin stain does not give consistently good results with smears. However, the method described by Tuan[7] (1930) gives a well differentiated stain and it avoids the long immersion in an aqueous solution. The following schedule is recommended by Tuan. Mordant in 2% iron alum for 20 minutes, wash in water 10 minutes, stain in 0.5% hematoxylin for 20 minutes, wash 10 minutes, destain in saturated aqueous solution of picric acid for about 2 hours, and wash in water for 20 minutes. Dehydrate and mount as usual.

The various standard fixatives and stains can be modified with good results in many cases. For chromosome counts and general morphology of the chromosomes the time of fixation can be reduced to 10 or 15 minutes and it is possible to make a permanent preparation in less than an hour.

The smear technic has made possible the determination of chromosome numbers of a large number of plant species in the past few years. The permanent smears, because of the superior fixation, have added greatly to our knowledge of chromosome structure and behavior. This technic is so simple and gives such excellent results that it should be used whenever possible.

It is of course necessary to use the proper color screens and optical equipment for best results in studying the preparations. For this phase of the work the writer is referred to Belling's book on the use of the microscope.

[7]Tuan, H. Picric acid as a destaining agent for iron alum hematoxylin. Stain Techn., 5, 135–138. 1930. A new method for safranin differentiation. Stain Techn.. 5, 103-107. 1930.

TECHNIC OF PREPARING THICK SECTIONS OF MATURE PINE CONES

H. J. Lutz, *Pennsylvania State College*

Abstract.—**The technic developed consists in first embedding the cone in paraffin (melting point 56 to 58°C). The cone should be thoroly air-dried prior to embedding. Following quick and complete cooling, the position of the central axis of the cone is determined; it is then an easy matter to mark the exact planes thru which to make cuts for transverse, radial or tangential sections. The sections may be cut with either a hack-saw or a carpenter's hand-saw. The sections are then dressed down with sandpaper. Permanent exhibits may be made by coating the polished sections with white shellac.**

Curiously enough, the preparation of thick sections of mature pine cones appears to have attracted but little attention in the past. Lack of interest in thick sections of cone material may be partly due to the failure of botanists and others to recognize generally the high instructive value which thick sections may have, and partly to the difficulties involved in preparing the sections.

The writer has found the use of thick sections distinctly helpful in classroom work as an aid in acquainting students with the general features of cone structure. As Chamberlain[1] suggests, thick sections may..... "afford a kind of study which is too often neglected." Figures 1 and 2 show respectively, median longitudinal and transverse sections of mature digger pine (*Pinus Sabiniana* Dougl.) cones.

At the outset the writer anticipated little or no difficulty in actually cutting the cone sections. The problem was to devise some method for holding the cone parts in their natural position during the process of cutting and afterward. Ordinary embedding paraffin with a melting point of 56 to 58°C was found quite satisfactory. Cones to be embedded should be air-dried at ordinary room temperature for several weeks prior to treatment in order to reduce their moisture content. The embedding may be accomplished in any convenient sized vessel. The writer has used small tin pails for this purpose. Several relatively small cones may be embedded in one block, but large cones, such as those of *P. Sabiniana*, *P. Coulteri* (Coulter pine), or *P. Lambertiana* (Sugar pine), are more easily embedded separately. Paraffin heated to about 65 to 70°C is poured into the vessel containing the cone and is then quickly cooled in cold water or in ice water.

After cooling, the embedded cone is removed from the vessel and

[1]Chamberlain, C. J. Methods in Plant Morphology, 4th revised ed. University of Chicago Press. 1924.

Figure 1. Median longitudinal section of *P. Sabiniana*, ×.6. The seeds are lacking but their positions are indicated by the light areas of paraffin. In the photograph the exterior coating of paraffin has been blocked out using India ink.

the excess paraffin is cut away, leaving a layer about 1.0 to 1.5 cm. thick surrounding the cone. The cone is then examined carefully in order to determine the exact position of its central axis. With the position of the axis established, it is a simple matter to mark the exact planes thru which the various cuts should be made to produce transverse, radial or tangential sections. It is probable that a small band-saw, such as may be found in almost any wood-working shop, would be well suited for making the various cuts. The writer has used a hack-saw for cutting small cones and an ordinary carpenter's hand-saw for cutting large sections, such as those of *P. Sabiniana*.

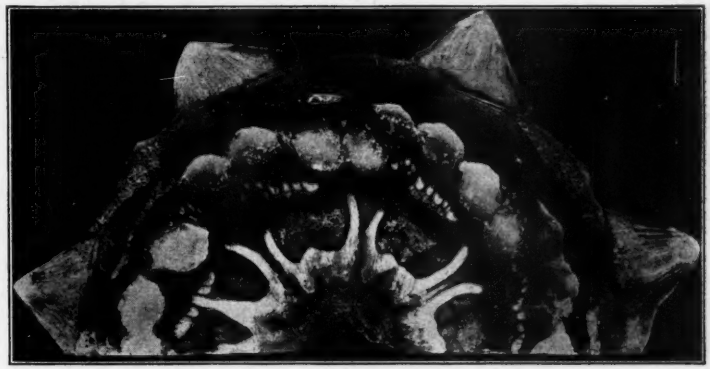


Figure 2. Transverse section of *P. Sabiniana*, ×.6.

After the sections are cut they are dressed down with fairly coarse sand-paper, and, finally, with very fine sand-paper. If a lapidary's lathe or wheel (a machine consisting essentially of a revolving lap on a vertical spindle) is available, the work of grinding and polishing the sections will be greatly reduced. Further, this apparatus makes it possible to secure a much higher polish on the sections than can be secured by ordinary hand methods.[2]

It is often desirable to photograph the finished sections and when this is done it has been found helpful to rub a small amount of oil over the surface before the photograph is made. This tends to bring out the detail. The oil used may be typewriter oil or any of the lubricating oils for general household use. The photographs for Figures 1 and 2 were made by using a Wratten "G" filter and panchromatic plates.

The sections may be converted into permanent exhibits if they are given one or two coats of any good quality of white shellac.

[2]The reader should refer to the note, "Conifer inflorescence" by G. R. Wieland in Science, 72, 113–115, 1930, for a fuller treatment of this phase of section making.

A TECHNIC FOR DEMONSTRATING PLASMODESMA

ALDEN S. CRAFTS[1]

Laboratory of Plant Physiology, Cornell University, Ithaca, N. Y.

ABSTRACT.—**Sections of potato tuber and epidermis from veins on the dorsal side of the sunflower leaf have proved most satisfactory in demonstrating plasmodesma. Kill fresh sections 5 minutes in a solution of 0.75 g. KI, 0.50 g. iodine in 100 cc. H_2O. Swell 5 minutes in 10% H_2SO_4. Mordant 5 minutes in a solution of 1.25 g. KI, 1.00 g. iodine in 100 cc. of 5% H_2SO_4. Wash in 5% H_2SO_4 until iodine starts to fade. Stain in a mixture of 0.5% gentian violet solution in 5% H_2SO_4 made up to a deep green color. Wash in a solution of 30 cc. glycerin, 2 g. $ZnCl_2$, 0.2 g. iodine, and a bit of KI in 60 cc. of water. Brush with a camel's hair brush, wash and mount in the same solution.**

The usual class room demonstration of plasmodesma in endosperm does not give the vivid picture of protoplasmic continuity in plants which may be obtained by studying them in stained sections of freshly cut tissues. The following method gives clear preparations which, tho not permanent may be quickly made with consistent results.

The solutions are those used by Gardiner[2] in his modification of Meyer's method.

Killing Solution. Potassium iodide 0.75 g., iodine 0.50 g., water 100 cc.

Mordanting Solution. Potassium iodide 1.25 g., iodine 1.00 g., 5% sulfuric acid 100 cc.

Staining Solution. (*a*) 5% sulfuric acid. (*b*) Gentian violet 0.5 g., water 100 cc.

Mounting Solution. Glycerin 30 cc., water 60 cc., zinc chloride 2 g., potassium iodide, a trace, iodine 0.2 g.

To prepare killing and mordanting solutions mix and warm until the iodine has dissolved to the point of saturation. The mounting solution is also warmed a bit and a small crystal of potassium iodide added, which dissolves some of the excess iodine. The resulting solution should be a light tan color. All solutions containing iodine should be kept in brown bottles and placed in the dark when not in use.

[1]National Research Council Fellow in Botany.

[2]Gardiner, W. Methods for the Demonstration of "Connecting Threads" in the Cell Wall. Proc. Camb. Phil. Soc., 9, 504–512. 1898.

A very handy and satisfactory material for demonstration is the potato (Fig. 1). Thin sections of the tuber are placed in the killing solution for about five minutes; then they may be swelled for a few minutes in 10% sulfuric acid, mordanted five minutes, washed in 5% sulfuric acid until the iodine starts to leave the section (10 to 30 seconds) and stained on the slide in a drop or two of the staining solution. Penetration may be hastened by warming the slide. The staining solution is made by placing about 1 cc. of *a* in a small watch glass and adding *b* drop by drop until a deep green color is obtained.

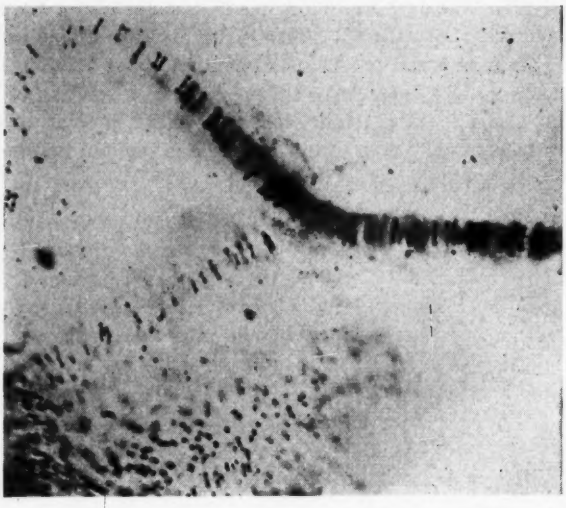


Fig. 1. Plasmodesma in the walls of the parenchyma cells of the potato tuber. × 750.

This solution should be used at once. As it fades more *b* may be added, but after it has been mixed for a half hour or more it should be discarded and mixed fresh.

When the sections have darkened, the stain is drawn off and a drop of mounting solution added. They are then brushed with a camel's hair brush on both sides to remove any precipitate present on the surface, and mounted in a fresh drop of mounting solution. The sections are good for several weeks and may be remounted in glycerin jelly or simply supplied with additional mounting solution until the glycerin has become concentrated. Brushing may be avoided by a proper combination of washing and staining but practice is required to judge these correctly.

The color of the staining solution is very important. If the sections come out with green cell walls and no plasmodesma showing, there is not enough *b* in the mixture, and if dark purple too much is present. The walls should be light blue or colorless with blue-black plasmodesma.

The pith tissue from the center of the tuber has given the most satisfactory results, the walls being thicker than in other parts. Thin tangential sections of a fresh onion are fairly good tho the cell walls are thinner and the plasmodesma shorter and not so numerous. Thin longitudinal tangential sections from the veins on the dorsal side of the sunflower leaf have given very good results. The sections are cut into tap water, mordanted for a few minutes, washed in 5% sulfuric acid, stained and mounted. Similar sections from tradescantia and cucumber leaves have been used but the pit membranes are thinner and do not show plasmodesma so clearly.

Transverse sections of the leaflets of *Cycas revoluta* make very striking preparations when treated by this technic. Small pieces of the leaf are killed over night in the killing solution, sectioned, and the sections swelled five minutes in 30% sulfuric acid and run thru the same schedule as the potato sections. Long plasmodesma show very clearly in the thick walled hypodermal cells and in the phloem of the midrib bundle.

The protoplasmic connections traversing the sieve plates of the phloem in cucurbits and potato have shown best when sections of stem killed in hot water[3] were treated with the killing solution or even with a more concentrated I-KI solution and then stained with 0.1% solution of water blue (water soluble anilin blue, Coleman & Bell Co.) and mounted in the mounting solution. By repeated alternate treatments an intensification may be effected and the long stretched connections in heavily callused sieve plates shown with clearness.

These methods may be modified for research but if followed carefully as given should yield good results in the hands of the average student of plant anatomy.

I wish to thank Miss Barbara McClintock for testing the gentian violet method.

[3]Fischer, A. Über den Inhalt der Siebröhren in der unverletzten Pflanze. Ber. Deutsch. Bot. Ges., 3, 230–239. 1885.

SOME EXPERIMENTS WITH SALTS OF HEAVY METALS AS FIXATIVES[1]

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Abstract.—**Heavy metal salts mostly in 0.5M concentration were used for tissue fixation and for albumin and gelatin precipitation. Tissues from dog, cat, and rabbit were stained in acid and basic stains. It was found that the atomic weight of the cation of the salt influenced its precipitating power. Penetration was either uniform or non-uniform and the resulting shrinkage generalized or cellular. Tissue hardening or high precipitating efficacy of a given salt did not always give good tissue preservation, but well preserved tissue was necessarily firm after fixation and fixed by a salt with a high precipitating efficacy. The mordanting effects of the fixatives were arbitrarily classified into three types: isomordants, basic mordants, and acid mordants.**

Introduction and History

In the past fifty years numerous investigations have been undertaken by both animal and plant cytologists and by chemists to gain information on the principles underlying microscopic technic and to ascertain the degree of faithful representation of cell-structure in fixed and stained tissue preparations. Sources other than those which deal directly with problems of technic such as precipitation, colloidal behavior of proteins, microchemical tests (McCallum, 1891, 1898; Nicholson, 1923–1924) of cellular structures, etc., have also yielded information, but the knowledge at hand is not sufficient to place the explanation of the mechanism of fixation and staining within limits of relative certainty, and more effort will be required to do so. The difficulties encountered in investigations of this type are no doubt due to the variety and complexity of the scientific fields involved and to the ever present, but equally variable "physiological state" of tissue.

From the standpoint of practical cytology, authors of leading textbooks on general or special techno-micrology (Hardesty, 1902; Mallory and Wright, 1915; Lee, 1924; Guyer, 1927; Romeis, 1928; McClung, 1929) agree on the fundamental requirements of good

[1]Contribution No. 162.

fixation. These requirements are best expressed by Schiller (1930) who states that a good fixative is one that changes the cell chemistry the least and preserves the cell structure the best. Little if any space in these textbooks is devoted to the discussion of the principles of fixation and its relation to staining. In the literature one finds that some investigations pertaining directly or indirectly to microscopic technic are of academic interest, while others are of practical value.

Fischer (1894, 1895, 1899), with the use of standard fixatives, precipitated a number of individual and mixed protein solutions in various concentrations. He colored the precipitates with cytological stains and imitated almost every conceivable cell-structure from an apparently homogeneous protein solution. This led him to distrust the reality of all the cell-structures appearing in cytological preparations. He observed also that proteins of apparently the same type and character were precipitated differently by the same fixing fluid. This phenomenon he interpreted in favor of the physical theory of precipitation as against the chemical one.

The interpretation of the mechanism of tissue fixation and cell-structure from observations made on similarly treated protein solutions is logical and, in a great measure, scientifically sound. The consensus of opinion (Hardy, 1899a, 1899b, 1900; Mott, 1912; Chambers, 1917; Bechhold, 1919; Bayliss, 1920, 1923; Loeb, 1922; Wilson, 1925; Bodansky, 1927; Mathews, 1930) is that living tissue is in a colloidal state of the suspensoid type which consists of a homogeneous liquid phase with ultramicroscopic particles suspended in it. Hardy (1899a) directed his efforts in search of the cause and mechanism of aggregation (coagulation and precipitation) of these ultramicroscopic particles in colloidal solutions. He stated: "In the formation of an insoluble modification of a colloid from a soluble form, there is a separation of the solid from the liquid, so that the particles of the former adhere to form a frame-work which holds the liquids in its interstices." He formulated, from his own work and from those of Schultze, Prost, and Picton and Linder, the conditions which determine coagulation as follows:

1. The point at which coagulation occurs is determined by the concentration of the solid in the colloidal mixture, the temperature, and the molecular concentration and nature of the electrolytes which are present.

2. The coagulation energy of electrolytes as measured by the number of gram-equivalents per litre necessary to produce coagulation is determined almost solely by the nature of the metal of the

salt and among the metals themselves it is determined by the nature of the metal.

3. If two electrolytes are present, then the action is additive if the metals are of the same valence, and subtractive if of different valence.

In studying the effects of fixing agents upon a pre-existent net of an insoluble colloid, Hardy found that there was generally no marked alteration of this net. At about the same time (Hardy, 1899b, 1900) he experimented electro-chemically with protein solutions and demonstrated the amphoteric nature of heat-modified proteins. He observed that when a current was passed thru an alkaline solution of protein particles, they moved from the cathode to the anode in the direction of the negative stream. After completing their transit to the coagulum on the anode, or after being in the anode region for some time, they altered so that they tended to reverse their movement and to travel with the positive stream toward the cathode. The reverse happened when the solution was acid in reaction. Very little movement of the protein particles was detected when a current was put thru a neutral solution, and he attributed this slight movement to the fact that the solution was not perfectly neutral. The latter observation led him to designate the electrical state of a protein at which it tended to move neither with the positive nor negative stream as the iso-electric one. As this point was neared, the stability of the hydrosol diminished until, at the iso-electric point, it vanished and coagulation or precipitation occurred. Whether precipitation or coagulation occurred depended upon the concentration of the protein, whether the iso-electric point was reached quickly or slowly, and the presence or absence of mechanical agitation. His paper of 1900 recorded precipitation experiments on proteins in acid and alkaline solution by heavy metal salts. He concluded that the coagulative power of a salt is determined by the valence of one of its ions; that this ion may be positive or negative; and that it is always opposite in sign to that of the colloidal particle.

Loeb (1922) came to the conclusion that the electrolytic behavior of proteins could be explained on a purely chemical basis. He reasoned that since proteins were amphoteric substances and slightly dissociable they would send H as well as OH ions into solution. When the particles sent more H ions than OH ions into solution they would have a negative charge, but a positive charge when more OH ions were given off than H ions. If acid were added to the solution in sufficient concentration the amphoteric colloidal particle would send more OH ions into the solution than H ions and hence assume a positive charge. The reverse would occur in an alkaline solution.

Therefore, a protein can be in either of three states: non-ionogenic or iso-electric; electro-negative (capable of forming metal proteinate); or electro-positive (capable of forming protein-acid salt). Proteins can not combine simultaneously with the cation and the anion of a neutral salt. Loeb did not attach much value to the Hoffmeister series in which ions are arranged according to their influence on swelling, viscosity, and osmotic pressure of proteins. He believed that the only difference among the influences exerted by ions, that was of any appreciable value was due to their valence.

According to Bayliss (1920), Clowes ascribed the differences observed between influences exhibited by mono- and bivalent ions of salts as due not to the valence of their ions, but to the opposition between the cation and anion of the given salt. Bayliss (1923) repeated some of Hoffmeister's experiments with slight modifications and observed variable influences exhibited by ions of the same valence on proteins. As against Loeb's purely chemical interpretation of the electrolytic behavior of proteins, Bayliss commented that his experimental data led him to conclude that it was more of a physical phenomenon of adsorption.

Concordantly with the enumerated studies one finds that a considerable amount of stress has been laid by investigators on problems dealing with isolated phases of technic, which are probably of more immediate value to the practical cytologist. These problems pertain to the effects exhibited by different fixatives on the preservation of cell structure, on shrinkage or swelling, and on staining of various tissues.

Donaldson (1894) made an extensive study of conditions governing weight, volume, and shrinkage of vertebrate brains, especially that of the sheep, during fixation and dehydration. He found that when a sheep's brain was put into a 2.5% aqueous solution of $K_2Cr_2O_7$ the increase in the weight of the brain would reach almost its maximum at the sixth day (34.2% increase). The greatest increase in the weight took place during the first day of fixation (17.8%). The amount of light or kind of water (distilled or tap) did not modify this increase, while temperature, concentration of salt in solution, and freshness of the tissues did effect it. The temperature favoring the highest increase was between 10° and 17°C. The increase in weight was in direct proportion to the dilution of the salt and freshness of the tissue. A significant observation made was that not all parts of the same brain, when identically treated, increase in weight to the same extent. This he believed was due to the different proportions of white and gray matter in the respective parts. In general the curve of volume

ran parallel to that of weight. King (1910) has not only evaluated the effects of commonly used fixing fluids on the weight of the brain of the white rat after fixation and after dehydration in 70% alcohol, but also studied thionin blue preparations of the cortex in celloidin and paraffin. She found: that there was a loss or gain in weight of tissue depending upon the type of fluid and length of time of fixation, and that the same was true after dehydration; that the cell picture varied greatly after different fixatives; and that celloidin embedding was the more preferable method, since, with all other conditions being equal, its usage resulted in superior morphological detail of the cells.

Variations in the length of embryos after fixation as compared with that before fixation were reported by Patten and Philpott (1925). The decrease in crown-rump length after fixation varied from 30% in 10 mm. embryos to about 20% in 20 to 25 mm. embryos. Additional data on shrinkage of brain and testicular tissue in the various steps of microscopic technic were given by Allen (1916) and on the spinal cord by Davenport (1931).

Type of fixation, as we have just seen, has a marked influence on weight, volume and lineal dimension of tissues. Its effects on the minute cytological structures are proved beyond doubt by the numerous highly specialized cytological procedures developed, some of which may be deduced from such work as King's (1910); Allen's (1916, 1919); Hopkins' (1924); and Zircle's (1927, 1928). The cytoplasm of the same type of cell, from the same tissue and animal, may appear as a diffused basophilic substance or it may have definitely formed bodies all depending upon the fixative—an observation brought out by Hopkins (1924). Zircle (1927, 1928) worked with several chromium salts and studied the effects of hydrogen-ion concentration upon fixation. He stated: "It is evident that there is an important relation between the pH of a bichromate and its fixation image. If it is too acid it will fix the chromatin but not the mitochondria, if it is too basic it will fix the mitochondria but not the chromatin".

Tolstoouhov (1927, 1928) has shown that the pH of the tissue and the staining characteristics are dependent on the type of fixing agent employed. By using various reagents on the same tissue he was able to determine the changes in the pH of the fixed tissue produced by each reagent.

Wilson (1925), after an admirable discussion of the composition and structure of protoplasm, stated: "There is reason to conclude that of all the cell constituents the 'structureless' hyaloplasm is the

most constant and the most active and may perhaps be regarded as forming the fundamental basis of the protoplasmic system from which, directly or indirectly, all other elements take their origin."

The present state of knowledge of fixation still offers numerous opportunities for investigation. The present work was designed to test what relation exists between the precipitating power, valence, and molecular weight of heavy metal salts on one hand, and tissue hardening, preservation, and staining on the other.

Material and Methods

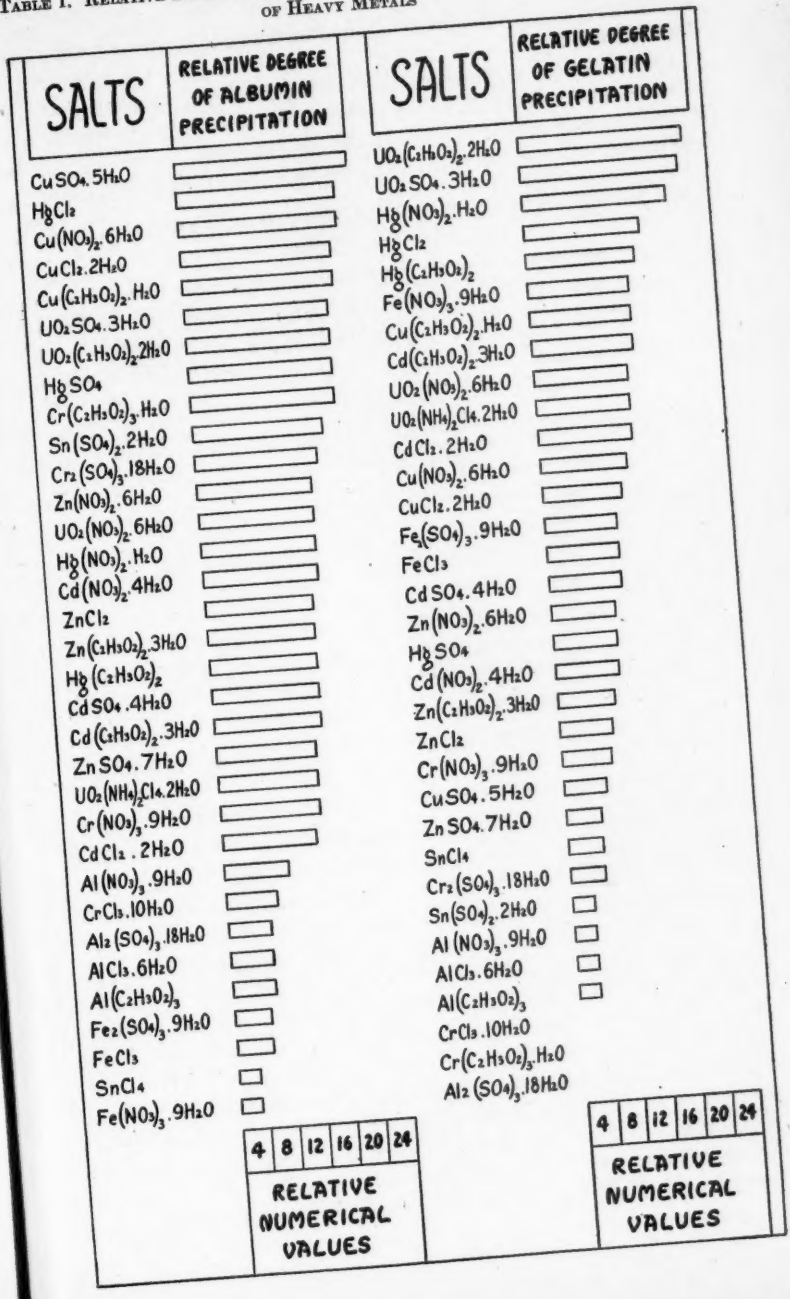
The salts used were in 0.5 M. concentration in water except those which formed saturated solutions before this concentration was reached. Preliminary tissue fixation experiments (not listed here) with some of these salts in concentrations ranging from 0.1 to 1.0 M. showed that the 0.5 M. gave the most favorable results, and for this reason this concentration was chosen.

I. *Precipitation.*—a. *Solutions employed.* Solutions of 0.1%, 0.5%, 1%, 5%, and 10% filtered fresh egg albumin and gelatin were precipitated at room temperature by thirty-three salts of heavy metals. Thruout this procedure, test tubes containing similar solutions, but no precipitating reagent, were used as controls. Not over five minutes were alloted for the appearance of the precipitate in the solutions tested. In each case the degree of precipitation was grossly observed, evaluated on a comparative basis according to the density and extent of the coagulum, and recorded by an arbitrary figure. The maximum degree of precipitation was designated by the value 6 and the minimum by 1.

b. *Method of determining the relative precipitating efficacy of a salt.*—The relative precipitating efficacy of each salt was computed by adding all the arbitrary values derived from testing the various dilutions of the given protein by this salt. Example: In precipitating 0.1%, 0.5%, 1%, 5%, and 10% of albumin by mercuric chloride the relative degrees of precipitation expressed numerically were 3, 4, 4, 5, and 6 respectively. Thus the relative numerical efficacy of albumin precipitation for mercury was the sum of the five individual tests, 22 (Table 1).

c. *Method of determining the relative precipitating efficacy of a cation.*—To obtain the relative numerical value of the mean precipitating efficacy of each cation, the numerical values of its salts were added and their sum divided by their number. Example: In testing albumin the numerical precipitating values of the mercuric salts were: acetate, 15; chloride, 22; nitrate, 16; sulfate, 20. The average

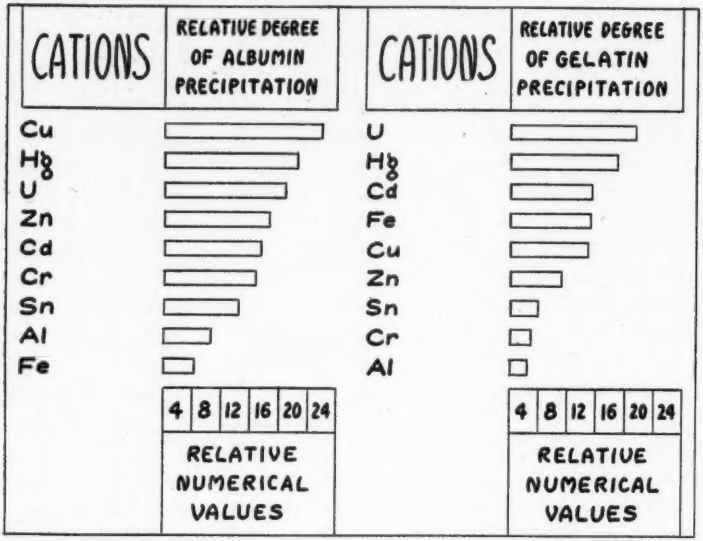
TABLE 1. RELATIVE DEGREE OF PRECIPITATION OF ALBUMIN AND GELATIN BY SALTS OF HEAVY METALS



numerical value for mercury as an albumin precipitant was $73 \div 4 = 18.25$ (Table 2).

d. *Method of determining the relative precipitating efficacy of an anion.*—The relative numerical value of the mean precipitating efficacy of each anion was computed by adding the numerical values of all salts of the same anion group and dividing the resulting sum by

TABLE 2. COMPUTED RELATIVE DEGREE OF PRECIPITATION OF ALBUMIN AND GELATIN BY CATIONS OF HEAVY METAL SALTS.



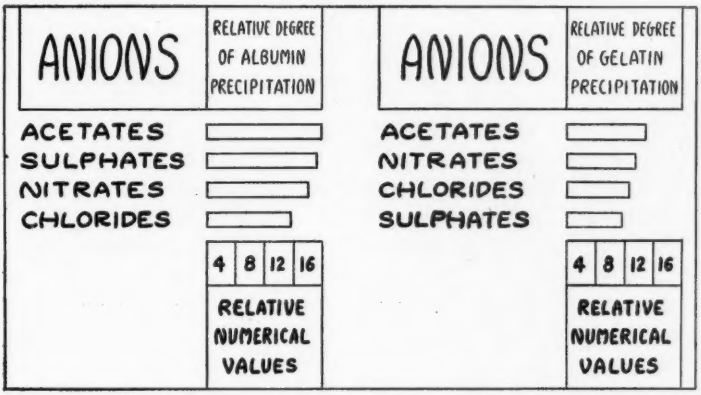
the number of salts employed. Example: In the albumin tests the arbitrary numerical values for the acetates were: Al, 6; Cd, 15; Cu, 21; Cr, 20; Hg, 15; UO_2, 20; Zn, 15. The average numerical value for acetates as albumin precipitants was $112 \div 7 = 16$ (Table 3).

II. *Fixation and staining.*—Spinal cord, cerebellum, liver or kidney, and stomach from adult cats, dogs, rabbits, and young kittens were fixed in each of the thirty-three heavy metal salt solutions for about one day. Specimens of the same tissues were fixed also in Zenker's fluid for comparison. In each case the degree of gross fixation (firmness) was determined by manipulation, and designated arbitrarily. Thus, the tissues showing the maximum hardening were designated as ++++, those showing the minimum as +, while those which failed to show any degree of firmness were regarded as degenerated and were discarded. After fixation the tissues were

dehydrated with alcohol, embedded in paraffin and cut into 10μ sections. One series of sections was stained with Mallory's triple connective tissue stain, another with a 1% aqueous solution of thionin blue, and still another with a 1% aqueous solution of orange G. The degree of tissue preservation was studied microscopically, and the type of microscopic penetration and shrinkage observed. The affinities for the stains were determined by gross comparison. Since there were 25 slides of each stain in the completed series the most intensely stained section was designated by 25, the least by 1.

III. *Hydrogen ion concentration.*—The approximate pH of the salt solutions was determined with the aid of indicators and a color chart.[2] The corresponding acid was added to some salts to keep them in solution. Seven per cent of sulfuric acid was required for $HgSO_4$. The pH of each salt was included in Table 4 merely for reference.

TABLE 3. COMPUTED RELATIVE DEGREE OF PRECIPITATION OF ALBUMIN AND GELATIN BY ANIONS OF HEAVY METAL SALTS



OBSERVATIONS AND DISCUSSION

It has been stated by Hardy (1899a) that the point at which coagulation occurs is determined in part by the molecular concentration and nature of the electrolyte, but no reference was made to its molecular weight. Table 4 shows that the molecular weight of a salt did not influence precipitation of protein solution since salts of nearly similar molecular weights differed greatly in their precipitating power. There is a strong indication however that the atomic weight of the

[2]Clark, W. M. The Determination of Hydrogen Ions. Reprinted from the third edition, 1928. The Williams and Wilkins Company, Baltimore.

TABLE 4. RELATION OF THE MOLECULAR WEIGHT OF THE ANHYDROUS SALT, ATOMIC WEIGHT OF METALS, AND APPROXIMATE pH OF SALTS TO ALBUMIN AND GELATIN PRECIPITATION, TISSUE HARDENING, TISSUE PRESERVATION, AND GROSS AFFINITY FOR BASIC AND ACID STAINS

Heavy Metal Salts in ½ M. Concentration	Molecular Weight of Anhydrous Salts	Atomic Weight of Metals	Approximate pH. of Salts	‡Relative Efficacy of Albumin Precipit.	‡Relative Efficacy of Gelatin Precipit.	§Degree of Tissue Hardening	Tissue Preservation	Gross ¶Affinity for Thionin-Blue Stain	Gross ¶Affinity for Orange-G Stain	Gross ¶Affinity for Mallory's Triple C-T Stain
$Al(C_2H_3O_2)_3$	204.17	27.1	†	6	3	+	Poor	1	2	1
$Al\,Cl_36H_2O$	135.54		3.0	6	3	+	Poor	5	1	2
$Al(NO_3)_39H_2O$	213.27		3.3	9	3	Tissue not fixed		–	–	–
$Al_2(SO_4)_318H_2O$	342.7		1.2	6	0	+	Poor	13	13	13
$Cd(C_2H_3O_2)_23H_2O$	230.5	112.4	5.8	15	14	Tissue not fixed		–	–	–
$CdCl_22H_2O$	183.3		5.6	13	13	+	Poor	12	6	6
$Cd(NO_3)_24H_2O$	236.5		3.9	16	9	Tissue not fixed		–	–	–
$CdSO4H_2O$	208.5		2.2	15	10	Tissue not fixed		–	–	–
$Cu(C_2H_3O_2)_2H_2O$*	181.6	63.57	5.3	21	14	++	Poor	8	17	19
$CuCl_22H_2O$	134.5		4.4	21	11	+	Poor	17	3	8
$Cu(NO_3)_26H_2O$	187.7		4.6	22	12	Tissue not fixed		–	–	–
$CuSO_45H_2O$	159.7		4.7	24	6	Tissue not fixed		–	–	–
$Cr(C_2H_3O_2(_3H_2O$	229.12	52.0	5.8	20	0	+	Poor	14	4	4
$CrCl_3lOH_2O$	158.54		2.6	7	7	Tissue not fixed		–	–	–
$Cr(NO_3)_39H_2O$	238.17		5.0	14	7	++++	Poor	4	24	18
$Cr_2(SO_4)_318H_2O$*	392.5		3.6	17	5	++	Fair	11	22	24

$FeCl_3$	162.6	5.84	1.8	5	10	++++	Fair	9	12	11
$Fe(NO_3)_3 9H_2O$	242.0		1.8	3	14	++++	Poor	2	23	23
$Fe_2(SO_4)_3 9H_2O$	400.0		2.0	5	10	++	Fair	6	25	25
$Hg(C_2H_3O_2)_2$	318.7	200.6	3.7	15	15	++++	Fair	25	20	17
$HgCl_2$	271.5		2.9	22	16	+++	Good	15	8	5
$Hg(NO_3)_2H_2O$	324.6		1.2	16	20	++++	Good	24	21	21
$HgSO_4$*	296.7		1.2	20	9	++++	Fair	16	19	22
$SnCl_4$	260.5	118.7	1.2	3	5	++	Poor	19	5	3
$Sn(SO_4)_2 2H_2O$	310.9		†	18	3	+	Poor	3	14	7
$UO_2(C_2H_3O_2)_2 2H_2O$	388.3	238.7	3.3	20	23	++	Poor	22	7	10
$UO_2(NH_4)_2Cl_4 2H_2O$	448.15		1.5	14	13	+	Poor	18	15	14
$UO_2(NO_3)_2 6H_2O$	394.3		2.2	16	13	+	Poor	7	16	15
$UO_2SO_4 3H_2O$	366.3		1.8	20	22	+	Poor	10	18	20
$Zn(C_2H_3O_2)_2 3H_2O$	183.5	65.38	5.5	15	8	++	Poor	21	11	16
$ZnCl_2$	136.3		2.0	15	7	+++	Fair	23	10	9
$Zn(NO_3)_2 6H_2O$	189.5		4.5	16	9	+	Poor	20	9	12
$ZnSO_4 7H_2O$	161.6		5.5	14	5	Tissue not fixed		–	–	–

*Saturated solution. †Hydrolyzed.

‡Expressed in numerical relationship: 24 being best albumin precipitant, 23 being best gelatin precipitant.

§Denotes degree of tissue hardening: ++++ being the tissue which was the most firm after fixation.

¶Relative gross affinity of the tissues for stains: 25 representing the greatest affinity for the given stain.

metal did exert such an influence. Uranium and mercury, with the highest atomic weights of the heavy metal series employed, seem to have been the most vigorous precipitants, while aluminum, with the lowest atomic weight, appeared to be the poorest. In addition (Table 1) it may be noted that different protein solutions did not react similarly to the same salt. $CuSO_4$ was a good albumin and poor gelatin precipitant; the reverse was true of $Fe(NO_3)_3$. Not all protein solutions were precipitated by all salts. This is demonstrated in Table 1. Fischer (1899) made use of a similar observation in his classification of fixatives. It is possible that failure of precipitation, at least in the course of these experiments, was due to the insufficiency of time alloted for precipitation (not over five minutes), or the precippitate was too fine and scarce to make itself macroscopically apparent.

The question arose whether the existing differences in the relative precipitating efficacy of a salt were due chiefly to its cation, its anion or to the entire salt molecule. The answer was attempted on a mathematical basis and computed from Table 1. Tables 2 and 3, thus constructed, show that it was due to a greater extent to the cations (Table 3) than anions (Table 2), for the variations between the anions, tho present, were relatively small as compared with those of the cations. This inference is strengthened by the previous findings (Table 4) that the degree of precipitation was in relation with the atomic weight of the metal (cation) of the salt. A few exceptions were encountered, as $HgSO_4$, for example, was a much poorer gelatin precipitant than $Hg(NO_3)_2$. This was due either to the anion, to the nature of the salt molecule, or to both. Since sulfates (Table 3) were relatively poorer precipitants of gelatin than nitrates it may be assumed that this can account at least in part for the variations exhibited by $HgSO_4$ and $Hg(NO_3)_2$. On the other hand it is possible that the nature of the salt molecule also had some bearing on it, as has been suggested by Hardy (1899).

Valence of the ions had apparently no marked influence on precipitation. In the case of albumin, Table 2 shows that anions of the same valence are at either extreme of the precipitating scale and from Table 3 one learns that not only did cations of similar valence react differently, but also that there was no apparent relation between their valence and position in the precipitation scale. This finding is not in agreement with Hardy (1899) or Loeb (1922) who believed that valence is one of the factors influencing precipitation of proteins. Therefore it may be concluded that, with all other conditions being equal, the principal single determining factor in the relative precipitating power of a heavy metal salt is the atomic weight of its cation.

Tissue hardening after fixation was caused mostly by precipitation, but swelling or shrinkage may have influenced it also. Donaldson (1894) has shown that tissue swelling during fixation is produced at the expense of both the solute and the solvent of the fluid. Since it is known that in precipitation the amphoteric protein, when not at or near the iso-electric point, may combine with either the cation, forming metal proteinates, or with the anion, forming protein acid salts and, since tissue hardening is mostly dependent on precipitation, it appeared logical to look for some relation between the two. The greatest as well as lowest degrees of tissue hardening were observed (Table 4) even when salts of the same metal, such as chromium, with nearly similar precipitating powers were used. The lack of

TABLE 5. CLASSIFICATION OF HEAVY METAL SALTS ACCORDING TO TYPE OF PENETRATION.

Uniform	Non-uniform
$Al(C_2H_3O_2)_3$	$AlCl_3$
*$Al_2(SO_4)_3$	$CdCl_2$
$Cr(C_2H_3O_2)_3$	$Cr(NO_3)_3$
$Cr_2(SO_4)_3$	$CuCl_2$
$Cu(C_2H_3O_2)_2$	$FeFl_3$
$Hg(C_2H_3O_2)_2$	$Fe(NO_3)_3$
$HgCl_2$	$Fe_2(SO_4)_3$
$Hg(NO_3)_2$	$SnCl_4$
$HgSO_4$	$UO_2(C_2H_3O_2)_2$
$Sn(SO_4)_2$	$UO_2(NH_4)_2Cl_4$
$Zn(C_2H_3O_2)_2$	$UO_2(NO_3)_2$
$ZnCl_2$	UO_2SO_4
$Zn(NO_3)_2$	

*Uniform only for spinal cord and cerebellum.

relation between precipitation and tissue hardening is shown by the behavior of some of the salts of cadmium and copper, for example (Table 4). On the other hand, there is evidence that the agents which produced a considerable degree of tissue hardening were also good precipitants, as illustrated by the mercuric and ferric salts. The lack of adequate correlation between precipitation and tissue hardening may be attributed in part to the type of precipitation taking place in the tissues, which in turn influences the ultimate intake of solute and solvent from the fixing fluid and in this manner effecting the hardening phenomenon. Still greater difficulties were experienced in the attempt to correlate precipitation with shrinkage. It was felt that this was due to the many ways in which shrinkage can be manifested. Most of the investigations on shrinkage deal with gross

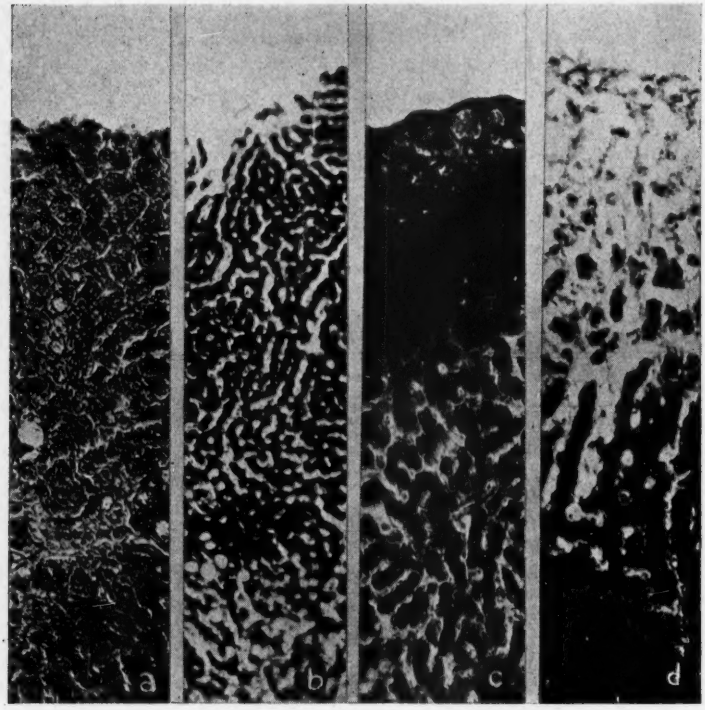


Fig. 1. Photomicrographs (extending from periphery to central part of tissue block) taken from 10μ sections of liver fixed in different heavy metal salt solutions and stained with Mallory's connective tissue stain. Magnification $\times$200.

a. Fixed in $Zn(NO_3)_2$. Penetration uniform, shrinkage generalized.

b. Fixed in $Cu(C_2H_3O_2)_3$. Penetration uniform, shrinkage cellular.

c. Fixed in $UO_2(C_2H_3O_2)_2$. Penetration non-uniform; peripherally shrinkage is generalized and results in compactness of structure; centrally shrinkage is more balanced in type.

d. Fixed in $UO_2(NH_4)_2Cl_4$. Penetration non-uniform; peripherally shrinkage is cellular and results in looseness of structure; centrally shrinkage is more balanced in type.

rather than microscopic dimensions. Microscopical study showed that the degree and type of shrinkage depended upon the penetrative ability of a salt and that salts could be classified approximately into two groups (Table 5): the uniform (a and b, Fig. 1) and the non-uniform penetrating (c and d, Fig. 1). Mercuric and zinc salts belong to the former, uranyl and ferric to the latter. Whatever the type of penetration, shrinkage (considering only the larger microscopic structures) may be either generalized, affecting all structures to a similar extent, or cellular, affecting primarily the cells. If penetration is uniform, shrinkage is either generalized (Fig. 1a) or cellular (Fig. 1b) thruout the block; if non-uniform, the type and degree of shrinkage varies in the peripheral and central parts. The peripheral part of the tissue block shows compactness of structure, if shrinkage is of the generalized type (Fig. 1c), and looseness, if of the cellular type (Fig. 1d). The central part in both cases shows a more balanced type of shrinkage. On the whole, shrinkage in most of the preparations was greater than that ordinarily observed in Zenker fixed material. Among the few exceptions encountered were $HgSO_4$, which was as good as Zenker's, and $FeCl_3$ and $Fe_2(SO_4)_3$, which showed less shrinkage in the anterior gray column cells of spinal cord than Zenker's fluid. The type of penetration and shrinkage could not be linked with molecular weight, or precipitating efficacy of the salt (Table 4) but it became apparent that not all histological components even of the same block of tissue shrink to a similar extent—a supposition made by Donaldson (1894) from variations observed in tissue swelling of similarly treated parts of the central nervous system of the sheep.

Evaluation of tissue preservation was based on the microscopic picture and included cellular and nuclear[3] shrinkage, regularity of the cellular and nuclear membranes, distinctness of the nucleolus, and cytoplasmic continuity as compared with Zenker fixed controls. The quality of a salt as a tissue fixative was evaluated and designated (Table 4) as "good", if its quality for all tissues was of the Zenker type; "fair", if only some tissues showed this quality; and "poor", if the quality of all tissues was distinctly inferior. It was thus found that of all salts used only $HgCl_2$ and $Hg(NO_3)_3$ were of Zenker quality. The question naturally arose whether there was any relation between the salt and tissue hardening on one hand and tissue preservation on the other. As for the salt, no relation could be found between its molecular weight, valence, cation, or anion and the

[3]Shrinking of nucleus away from cell body was observed in the Purkinje cells of the cerebellum after $SnCl_4$ fixation.

quality of the microscopic picture. Table 4 shows the following findings: 1) Salts of nearly similar molecular weights (such as those of mercury and uranium) produced histological pictures of diversified character; 2) Salts of metals of the same valence (mercury and copper) gave different structural images; 3) Salts of the same metal (zinc group) presented variable results; 4) Similar salts of different metals (chlorides of copper, iron, and mercury) have furnished nothing in common. Some idea about the lack of correlation between tissue hardening and tissue preservation may be obtained by comparing the data in the corresponding columns of the same table, but an inference may be made that while good tissue hardening, as seen after $Cr(NO_3)_3$, does not necessarily result in good tissue preservation, good tissue preservation must be preceded by good tissue hardening, as noted after $Hg(NO_3)_2$.

In the last three columns of Table 4 which deal with the comparative staining affinity of the salts, the following points may be noted: all the electrolytes in this series were endowed with some mordanting quality; tissues similarly to both, a simple and compound acid dye, as shown after fixation in $CdCl_2$, $Fe(NO_3)_3$, $HgCl_2$, etc.; after fixation in $Al_2(SO_4)_3$, $HgCl_2$; etc., some tissues exhibited equal (or nearly so) affinities for both an acid and a basic dye; some tissues fixed in $UO_2(C_2H_3O_2)_2$, $ZnCl_2$, etc., had a stronger affinity for the basic dye, while others were more favorable to the acid dye as shown after fixation in $Fe(NO_3)_3$, $Fe_2(SO_4)_3$ etc. These differences between the affinities were most probably due to the variable degree of acidity of the salts employed. This in turn influenced the iso-electric point of the tissue and, as has been shown by Tolstoouhov (1927, 1928) and Zeiger (1930), determined its affinity for the stain. On the basis of the presented evidence it is proposed to identify mordants of three kinds:

1. *Isomordants*, with a similar affinity for both acid and basic dyes.
2. *Acid mordants*, with a greater affinity for acid dyes.
3. *Basic mordants*, with a greater affinity for basic dyes.

Whatever the mechanism of fixation and staining it is obvious that, with all other conditions being equal, the type of the fixative employed greatly influences the ultimate staining of the tissue. This agrees with the findings of Naylor (1926), Tolstoouhov (1927, 1928), and Zeiger (1930).

Summary

The present investigation, tho it failed to answer many of the questions raised in the discussion, has nevertheless shown the following:

1. Precipitation of protein solutions is generally influenced by the atomic weight of the cation of the electrolyte.

2. A relatively good protein precipitant is not necessarily a good tissue hardening agent, but the reverse holds true.

3. Penetration of an electrolyte may be uniform or non-uniform and the resulting shrinkage in the former may be either generalized or cellular, in the latter, usually both.

4. Tissue hardening is no criterion for evaluation of tissue preservation, while the acquisition of a favorable histological picture must be preceded by good block hardening.

5. Mordants can be classified into: isomordants, acid mordants, and basie mordants.

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LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

ANTHONY, E. E. A note on capsule staining. Science, **73**, 319–320. 1931.

This method is more dependable than that of Hiss. Stains tested for it were crystal violet (dye content 84%), crystal violet (dye content 92%), crystal violet (dye content not available), methyl green, methyl violet 1 B, methyl violet 2 B, (two brands), and anilin violet. Names of the brands are not mentioned for any of these dyes.

Methyl green was not satisfactory in any of the procedures tested. All violets were more or less satisfactory, but best results were obtained with 1% aqueous crystal violet, 84% dye content. Method: Stain air-dried, undiluted smears for 2 minutes. Wash in 20% copper sulfate and dry. An increase in the time of staining did not improve the picture.

Materials used were pneumococci from the peritoneal cavity of an infected mouse and *Klebsiella pneumoniae* from serum agar slants. The procedure omits heat, is fast and does not fail even in class use.

CROUGH, H. B. and BECKER, E. R. A method of staining oocysts of Coccidia. Science, **73**, 212–213. 1931.

This method facilitates the study of internal structures of spores inside the mature oocysts, otherwise impossible due to their extremely refractive nature.

Strain the fecal material thru a double layer of cheesecloth. Centrifuge in ordinary pointed tubes, add more water and repeat twice, to remove most of the débris. Add concentrated salt solution, shake well and centrifuge. The oocysts will appear on the surface; collect and transfer them to slide and cover with a cover glass. Replace the salt solution with glacial acetic acid by drawing it with the help of blotting paper under the cover glass. Most of the oocysts are held in place by contact with the glass above and below. Gently warm the slide for 5 to 10 minutes; do not evaporate the acid.

Replace the acid by a freshly prepared 0.01% aqueous solution of Janus green in the above-described manner, stain 10 minutes. Wash and stain with a concentrated solution of aqueous eosin for 5 minutes. Wash, blot off excess water and seal with amber vaseline or glycerin jelly. This whole process may be watched under low power. Studying is recommended under oil immersion.

The oocyst jelly stains red, the walls may appear reddish, and structures within the sporozoites are rendered visible.

FARRIER, R. and WARTHIN, A. S. A study of the effect of pH upon the third improved Warthin-Starry method for demonstrating Spirochaeta pallida in single sections. Am. J. Syph., **14**, 394. 1930.

This is a modification of the Warthin-Starry method, stressing the importance of a suitable reaction for successful silver impregnation. Fix tissue in formalin, embed in paraffin and make cover glass preparations. Run thru xylol and alcohols to water. Using brom cresol green as indicator, prepare a liter of water of pH 4.4. Prepare a 2% silver nitrate solution from this adjusted water and dilute a part of it to 0.5 to 1%.

Pour enough of this diluted solution into a perfectly clean bottle so that when the cover glass is placed in it on edge the solution will cover the section by capillary attraction. Rinse in the adjusted water and place in the silver solution, with the section side facing the side of the bottle.

Impregnate in darkness at 37°C. for 30 minutes and transfer to small dish, section side up. Add 15 cc. of a 5% gelatin, heated to 45°C, to 3 cc. of 2% silver nitrate. Mix

and add 1 cc. of 5% hydroquinone, mix rapidly and pour over the section. Reduce until the gelatin begins to turn brownish-black. Rinse and place in 5% sodium hyposulfite for few minutes. Wash, pass thru 96% alcohol, two changes of absolute, then xylol, and mount.

FOLEY, J. O. Studies in stain technic. III. A cytological method for staining with the Ehrlich-Biondi mixture. Anat. Rec., **49**, 15–17. 1931.

This is a modification of the Ehrlich-Biondi stain, by means of which various tissues and cytologic details are revealed. Method: Fix in Allen's B 15 without osmic acid or in the usual Flemming's strong solution. After the removal of the paraffin pass sections thru potassium permanganate and oxalic acid and wash in running water. Mordant in 0.5% osmic acid if none was present in the fixative used. Wash.

Prepare the following aqueous solutions: 0.23% methyl green; 0.11% acid fuchsin; 0.1% orange G. These stains were Coleman and Bell's products, with actual dye content given. Before using, mix in the following order: glycerin 10 cc.; acid fuchsin 20 cc.; orange G 30 cc., and then, slowly and with constant stirring, methyl green 30 cc.; and stain therein 12 to 24 hours.

Wipe off excess stain and blot lightly. Immerse in 95% acid alcohol for 5 to 30 seconds depending on the desired nuclear stages. Transfer to carbol-xylol, xylol and mount.

Results obtained with gonads of the mud minnow and white mice were as follows: compact chromatin light green, less densely arranged chromatin blue green, collagen bundles red, and the cytoplasm brownish-yellow.

HERTERT, L. D. Differentiation of the various types of fats by means of dyes. J. Lab. & Clin. Med. **16**, 926–929. 1931.

Nile blue sulfate is recommended for broader application by pathologists since it differentiates between various fats, which sudan III, and scharlach R (sudan IV) fail to do.

For the examination of stools use: Nile blue sulfate (National Aniline & Chemical Co.) 0.1 gr.; glacial acetic, 1 cc.; distilled water, 100 cc. Fatty acids stain a deep Nile blue, except oleic acid; neutral red stains a deep red, approaching garnet; intermediate compounds a violet purple or magenta. Cholesterol crystals stain as irregular blue rhomboids. A light Nile green is produced by a combination with plant or meat fibers, but more frequently these appear a deep blue; nuclei, if present, are sharply outlined. Unfortunately crystals of fatty acids may also appear green. Neutral fats may be masked by fatty acids.

Huepke's method may be used for check. Mix a small piece of stool with few drops of a saturated solution of cupric nitrate; heat, but do not boil. During this procedure, metallic green copper soaps are formed with fatty acids. Add a drop of 0.25% dimethyl-amino-benzaldehyde. Neutral fats stain a brilliant yellow with the latter reagent. If the stool is too acid and the red color of the benzaldehyde obscures the yellow fat globules, neutralize with a little NaOH. Huepke advises Orleans as a counterstain in instances of hyperacidity.

A combination of the two technics lends almost as much information as quantitative examination of stools for the various fat fractions.

HORVATH, P. Sublimat-Toluidinblau für Cilienfärbung. Zts. Wis. Mikr., **47**, 463–465 1931.

This procedure is based on Gelei's osmium toluidin blue method. It has an advantage over the latter in giving a more uniform and permanent staining, and is cheaper because osmic acid is dispensed with.

Centrifuge the material, pipette off the lower part and drip it into concentrated $HgCl_2$; do not pour the sublimate on the organisms directly, for this results in poor fixation. After 10 to 20 minutes, wash in water.

Mordant in 0.33 to 1.65% phosphotungstic acid for 10 to 15 minutes. Discard the mordant after it has been used. Wash thoroly in water.

Stain in toluidin blue (source not mentioned) at 50 to 60° C for 30 seconds to several minutes. Use a 1:1000 dilution for Holotrichida and 1:5000 for Hypotrichida. Pass thru 30%, 50%, 70% and 96% alcohols, absolute alcohol, three changes of xylol and mount in balsam. All this is done by centrifuging.

If organisms are mordanted after the sublimate-osmium fixation the staining is also good, but such preparations are not permanent, for they fade.

Results: light blue cytoplasm; dark blue cilia, cirri and basal bodies.

KEDROVSKY, B. Anwendung von heterodispersem Eosin zur Färbung histologischer Präparate. Zts. Wis. Mikr. **47**, 433–442. 1931.

The importance of colloids in the selective stainability of certain structures is stressed. In connection with this physical theory of staining a method is offered where a hetero- or poly-dispersed eosin is used. It is a modification of the Dominici's procedure.

Fix in Zenker's, Helly's or neutral formalin (the latter for sheaths of connective tissue). Embed in celloidin paraffin. Stain sections for 20 to 30 minutes in a solution prepared as follows: To 15 cc. of a boiling 0.6% eosin solution (eosin reddish, König or Grübler), add 0.5 to 1 cc. of 1% phosphomolybdic acid (whereupon the solution will turn yellowish and become cloudy); then add 15 cc. of 1.2% cold aqueous orange G. (The mixture does not keep over 3 to 4 days.) Counterstain in a 1% aqueous solution of toluidin blue for 0.5 to 1 minute and rinse. Differentiate slowly in 70% and 96% alcohol, controlling under the microscope. Pass thru absolute alcohol, bergamot oil, and xylol; then mount in balsam.

Rose bengal and eosin B A may be employed instead of the eosin used.

Results: chromatin dark blue or lilac; nucleoli bright red to orange; cytoplasm varies from blue to lilac, reddish or colorless. Spindles are selectively stained; centrosomes (of *Ascaris*) rose to lilac; muscles red; spinal cord nucleoli red, Nissl's substance blue; in blood, eosinophilic granules red, basophilic violet; basophilic plasma bluish lilac, not characteristic; erythrocytes red.

KEMP, H. A. A simple stain for metachromatic granules. J. Lab. & Clin. Med., **16**, 593. 1931.

This procedure is preferred to Albert's or Neisser's stain for polar bodies in bacteria. Prepare smears from throat cultures as usual. Flood with Gram's iodine for 1 minute, rinse in tap water; stain with Loeffler's methylene blue for 20 to 30 seconds; rinse; stain with 1% aqueous safranin for 10 to 15 seconds, rinse, dry and mount. (Source of stains not mentioned.)

The method was tested out and found successful for class work at Baylor University and at the hospital of the same university. The advantage of this stain is in the contrast of color between the polar bodies and the body of the organism. The polar bodies are dark bluish-black, while the organism is red. Other organisms stain red. The Gram-positive cocci seem to stain darker than other bacteria.

LÖTE, v. J. Reflexionen über die Sporenfärbung. Zentbl. f. Bakt., I Abt., Orig., **120**, 131–135. 1931. Über die Färbung der Tuberkelbazillen auf Grund des Farbentaushes. Idem. 135–139. 1931.

Altho these articles deal with different subjects they discuss the same principles and methods and one abstract may well cover both. The author discusses the staining of spores and of the tubercle organism. He stresses the importance of heat in both processes. Smears may be stained without heat if heated previously for one hour at 100°C, cooled and flooded with the stain for a minute or two. Acids used for differentiation are undesirable, as they may damage the organisms and tend to decolorize the spore capsules.

The following procedure is recommended: Steam 1 to 2 minutes with carbol fuchsin, rinse, dry and differentiate with 1 to 1.5% methylene blue. Spores of *B. anthracis* require 10 minutes, those of the potato bacillus 5 minutes, and the tubercle organism 30 to 60 minutes for differentiation. Spores and tubercle bacteria are red, other bodies bluish-purple, greyish or blue. Bismarck brown (0.5 to 0.25%) may be used instead of methylene blue. Carbol-methylene blue may be used instead of the fuchsin as in the Ziehl-Neelsen technic in staining the tubercle organism. Bismarck brown (0.25 to 1%) applied for 3 to 6 minutes contrasts well with methylene blue. Eosin (1%) may be used too, which destains within 20 to 30 minutes. The tubercle bacteria should be blue amidst other brownish or pink bodies.

MATERNOWSKA, IRENA. Eine neue Färbungsmethode zum Nachweis der Abortus-Bang Bakterien im natürlichen faulenden Material. Zentbl. f. Bakt., I Abt., Orig., **116**, 422. 1930.

This method is of special diagnostic value for the abortion organism when decomposition products and other bacteria obscure the picture. Small pieces of well preserved placenta are thoroly washed to isolate the external foul masses. Smears obtained from this cleansed material almost always contain *Bacterium abortus* Bang.

Fix smears and apply 10% potassium hydroxide for 5 minutes. Stain with a 2% solution of carbol-thionin (no information given as to source of stain or dye-content) for 3 to 5 minutes, rinse and dry.

Potassium hydroxide not only loosens the epithelial cells, but intensifies the stainability of the organism in question and this facilitates diagnosis.

McNaught, J. B. Suggestions for hanging drop preparations. J. Lab. & Clin. Med., 16, 620. 1931.

Dip an ordinary lipped glass tube in melted petrolatum and then touch with it the surface of a slide, leaving a smooth ring. Invert the cover glass containing the material to be studied, press gently and the preparation is sealed.

For a true hanging drop draw melted petrolatum in a Wright's pipette and by pressing the nipple inscribe a circle of desired size on the slide.

Still simpler is the use of tubed petrolatum jelly. In this case a hole is punched and the rest is done as above.

Vrtis, V. Über elektive Färbung der Talgdrüsen mit Sudan III in den Totalpräparatene der Haut. Zts. Wis. Mikr., 47, 443–450. 1931.

This is a method for the total staining of fat glands, tested mainly on rodents. Altho it naturally does not replace the use of sections for detailed study, it is very valuable for reconstruction purposes. Proceed as follows: Fix portions of skin or whole small skins in 10% formalin, wash in water and for 15 to 30 minutes in 50% alcohol. Stain 30 to 45 minutes in a saturated solution of sudan III (Grübler's) in 70% alcohol. Differentiate in 50% alcohol for 30 minutes to several hours, rinse in water and transfer to glycerin. The objects clear sooner or later, depending on their thickness. Preparations mounted in glycerin and sealed with Kitt wax have not faded for three years. Fat glands, their outlets and branches appear yellowish red while all other tissue are more or less transparent.

Pigments present in the neighborhood of glands are usually readily distinguishable from the latter. Chemical depilators have been tried instead of the tedious cutting of hair previous to fixation but proved unsuccessful for they attacked fat before destroying hair.

Nile blue sulfate is not suitable for this method. Borax carmin gives very precise pictures of fat glands, etc., but it is not specific and other cells or elements obscure fat organs. Weak glycerin solutions of hematoxylin give results similar to those obtained with borax carmin. Sudan III remains, therefore, the most highly recommended stain for fat glands.

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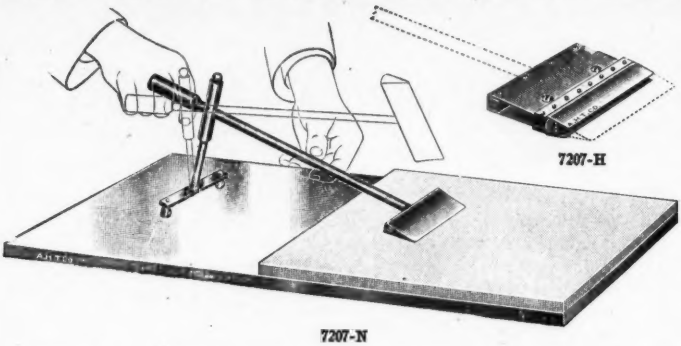

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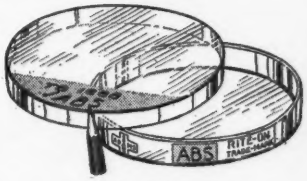

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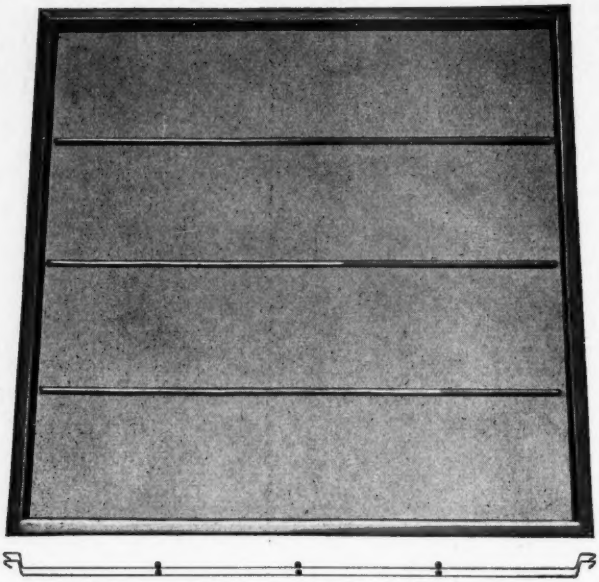

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